PATHOGENESIS OF DENGUE IN THE DEVELOPMENT OF HEMORRHAGIC FEVER

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ABSTRACT

Prior research has shown enhanced uptake of dengue virus by monocytes in the presence of immune serum containing anti-dengue antibodies. This is the basis of the antibody-dependent enhancement (ADE) theory, a major hypothesis which has been proposed to explain the pathogenic mechanism of DHF. An in vitro ADE model was established in this study by using flow cytometry to detect dengue 2 infection of the monocytic cell line U937. Flow cytometry also was used to observe TNFα, IL-8 and IL-10 production by dengue 2 infected U937 cells incubated with and without dengue immune serum. Cytokine RNA expression was also analyzed in dengue infected U937 cells. Enhancement of dengue virus was observed in cultures incubated with immune serum. A significant increase in IL-8- and a slight increase in TNFα-producing cells were seen in infected cultures incubated with immune serum. Cytokine protein and RNA obtained via flow cytometry and RT-PCR did not correlate, however the hierarchy of cytokine expression in dengue-infected cells was similar. An in vitro vascular permeability model using dermal microvascular endothelial cells in a Transwell was developed to evaluate the permeabilizing activity of dengue 2-infected cell supernatants. A horseradish peroxidase (HRP) assay was used to measure monolayer permeability. Permeability changes were observed in monolayer cultures incubated with culture supernatants of virus-infected monocytes. The approaches developed in this study will be useful in future studies to evaluate potential mechanisms of dengue immunopathogenesis.
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADE</td>
<td>antibody dependent enhancement</td>
</tr>
<tr>
<td>BBS</td>
<td>borate buffered solution</td>
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<tr>
<td>cDNA</td>
<td>copy of deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<tr>
<td>DV</td>
<td>dengue virus</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HI</td>
<td>hemagglutinin inhibition</td>
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<td>HR</td>
<td>hour</td>
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<tr>
<td>HSBBS</td>
<td>high salt borate buffered solution</td>
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<td>IgG</td>
<td>immunoglobulin</td>
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<td>IL-8</td>
<td>interleukin 8</td>
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<tr>
<td>IL-10</td>
<td>interleukin 10</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>μL</td>
<td>microliter</td>
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<td>ml</td>
<td>milliliter</td>
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<tr>
<td>MOI</td>
<td>multiplicities of infection</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>PBS</td>
<td>phosphate borate saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Pfu</td>
<td>plaque forming unit</td>
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<td>Abbreviation</td>
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<tr>
<td>PRNT</td>
<td>plaque reduction neutralizing test</td>
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<tr>
<td>rpm</td>
<td>rotations per minute</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain</td>
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<td>TNFα</td>
<td>tumor necrosis factor</td>
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Dengue viruses (DV) are single-stranded, positive sense, lipid-enveloped RNA viruses that belong to the family *Flaviviridae*. There are four antigenically distinct serotypes, dengue virus types 1 to 4, which are transmitted to humans primarily by the mosquito vector *Aedes aegypti*. Other mosquito species such as *Aedes albopictus* and *Aedes polynesiensis* have also been implicated in dengue transmission. Infection with any of the four serotypes of dengue virus can cause self-limiting dengue fever (DF) or the severe dengue hemorrhagic fever (DHF) and potentially fatal dengue shock syndrome (DSS). Dengue is one of the most important human viral diseases transmitted by arthropod vectors as it is estimated that there are 50-100 million cases of dengue fever, and 250,000 to 500,000 dengue hemorrhagic fever cases annually with the majority of dengue cases occurring in the tropical areas of Africa, Asia, and the Americas (Rigau-Perez *et al.*, 1998). Epidemics of dengue hemorrhagic fever have become more frequent in the past 50 years and have also become a leading cause of pediatric morbidity and mortality in some Southeast Asian countries.

Infections with dengue virus can range from being asymptomatic to producing a broad range of clinical presentations, including death. The incubation period ranges from 3-14 days and typical clinical manifestations of dengue fever (DF) include fever (5-7 days), headache, bone/joint pain, rash, nausea, and vomiting. In some cases, patients develop the life-threatening dengue hemorrhagic fever (DHF), which is characterized by increased vascular permeability, severe thrombocytopenia, plasma leakage, hypoalbuminaemia or hypoproteinamemia, and shock. The severity of DHF is
subgrouped according to the following clinical criteria: Grade I: a positive tourniquet test (which evaluates resistance of capillary walls to pressure); grade II: spontaneous bleeding, in addition to manifestations of grade I; grade III: circulatory failure with rapid, weak pulse or hypotension; grade IV: no detectable blood pressure and pulse. Grades III and IV are also known as dengue shock syndrome (DSS). Thus, DF and DHF/DSS are not separate clinical entities, but rather a progression of the disease (McBride et al, 2000).

The major pathophysiologic hallmark that determines the severity of DHF is the plasma leakage due to the sudden increase in vascular permeability, leading to hemorrhage. Past studies have suggested that DHF is induced by immunopathological mechanisms that involve both humoral and cell-mediated immune responses (Rothman et al, 1999). Although the pathogenesis of this increased vascular permeability has yet to be determined, there are a couple of theories that have been documented. A long-standing hypothesis referred to as “antibody-dependent enhancement” (ADE), proposes that preexisting nonneutralizing antibodies enhance DV infection of monocytes via Fc receptors. The immune enhancement phenomenon occurs in the following basic steps (reviewed by Gubler, 1997):

1. An initial infection with DV occurs and produces antibodies.
2. A subsequent infection with a different serotype occurs.
3. The second virus complexes with the pre-existing, cross-reactive, non-neutralizing antibodies made from the initial infection.
4. The virus-antibody complex enters monocytes via Fc receptor.
5. When the virus-antibody complex enters the monocyte, it allows the virus to enter the cell without any immunological attack. Thus, once the virus is in the cell, it may replicate and increase its viral load, which may result in exponential growth of virus particles within the body.

Newly synthesized dengue viral antigens in the target cell are presented via MHC antigens which can lead to T-cell activation. One of the consequences of this activation is increased cytokine production, which activates other cells, resulting in a chain reaction and the release of more and/or additional cytokines. Ultimately, this cytokine cascade may produce an increase in vascular permeability which depending on the severity may lead to hemorrhage in severe cases.

In addition to the possible involvement of monocytes and T cells, B lymphocytes have also been found to produce high titers of anti-platelet and anti-endothelial cell autoantibodies, which could then contribute to pathogenesis by inducing coagulopathy and vasculopathy, two major pathologies of DHF/DSS patients (Lin et al., 2002).

Another theory suggests that viral virulence factors may play the key role in the pathogenesis of DHF/DSS (McBride et al., 2000). Possible roles of viral virulence factors may encompass the ability of viruses to (1) infect more cells, (2) generate more progeny virus, (3) cause a more severe inflammation, and (4) evade host immune responses.

Regarding the role of cytokines in pathogenesis, a recent study by Chaturvedi et al., 2000 has shown that a shift from a predominant Th1 type response in DF cases to Th2 type in DHF may be responsible for the increased severity of disease in DHF/DSS patients. Specific cytokines that have been documented as being elevated in DHF patients include IL-1, IL-4, IL-6, IL-8, IL-10, IL-12, TGF-β, TNF-α, and hCF, a unique
cytokine produced in dengue infections. When released by cells such as macrophages or neutrophils, cytokines such as TNFα and IL-1 have been shown to activate vascular endothelium resulting in an increase in vascular permeability and vasodilation leading to loss of plasma volume along with the migration of cells out of blood vessels.

The two major pathophysiologic changes that occur in DHF/DSS are (1) plasma leakage from the vascular compartment and (2) hemostatic impairment, both involving the injured/activated endothelium. Potential factors as TNFα and IL-1 have been shown to have multiple effects on endothelial cells including suppression of surface anticoagulant activity, enhancement of procoagulant activity, and increased permeability (Chen et al, 2000). Thrombocytopenia is often seen in DHF infections and this may be due to decreased platelet production or increased platelet consumption. Platelet dysfunction, as well as diminished levels of plasma coagulation factors (e.g. fibrinogen, plasminogen, and prothrombin), has also been associated with DHF/DSS.

Monocytes are believed to be a primary target cell for dengue virus replication and probably modulate the containment and/or spreading of viral infection into other cell types. Monocytes also play a significant role in the induction and regulation of immune responses and hemostasis, through the synthesis and excretion of soluble mediators such as cytokines (TNFα) and chemokines (IL-8) (Chen et al, 2000). Since there is evidence of minimal vascular endothelial cell damage from dengue virus infection, circulating mediators are believed to be mainly responsible for the vascular leakage and hemorrhage seen in DHF/DSS. TNFα has been shown to upregulate tissue factor expression on the surfaces of monocytes and endothelial cells (e.g. adhesion molecules). TNFα is also an effector capable of disrupting and destroying the endothelial monolayer, however clinical
evidence argues against endothelial damage during dengue hemorrhagic disease (as mentioned above), as the cause of increased vascular permeability. Dengue infected monocytes have been shown to activate endothelial cells in vitro, which involves TNFα and IL-1β (Bosch et al., 2002). IL-1 also can increase tissue factor expression on the surface of endothelial cells and synergize with TNFα to produce inflammation.

The endothelial cells that line the vessels also secrete soluble mediators. In vitro studies have shown that endothelial cells infected by dengue virus produce IL-8, MCP-1, RANTES, as well as activate complement. Dengue infected monocytes have been shown to activate endothelial cells, by a mechanism involving TNFα and IL-1β (Bosch et al., 2002), and can induce IL-8 transcription in endothelial cell lines. Cross-reacting, non-neutralizing antibodies to DV have been found to activate complement on the surface of infected endothelial cells, causing liberation of anaphylatoxins (Avirutnan et al., 1998).

Upregulation of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin (Anderson et al., 1997) was also found and are indicative of endothelial cell activation.

The supernatant of dengue-infected macrophages has been shown to induce increased permeability in endothelial cells (Carr et al., 2003).

Other diseases or abnormalities that induce enhanced endothelial permeability include diabetes mellitus, atherosclerosis, pre-eclamptic condition, anaphylactic shock, septic shock, and certain bacterial/viral infections. A common complication of diabetes mellitus includes basal lamina thickening and endothelial cell proliferation of blood vessels which may lead to retinal and renal damage, as well as accelerated atherosclerosis. Damage to the endothelium occurs in the early stages of atherosclerosis, leading to an increase in permeability that allows platelet deposition and entry of lipids.
such as cholesterol into the intima. The normal endothelium acts as a barrier to prevent entry of macromolecules including lipoproteins from the blood into the arterial wall (Taussig et al, 1984). Pre-eclampsia a condition in pregnancy characterized by hypertension, proteinuria, and edema is also related to disruption of the endothelium. A substance known as factor x which the “sick” placenta releases into the mother’s blood stream may play a role in this condition as factor x appears to cause damage to maternal blood vessel endothelium. One of the main effects it has is making the maternal blood vessels leaky, thereby allowing fluid to seep into the tissues. In severe cases the glomeruli of the kidney are also involved and become enlarged due to marked swelling of the endothelial cells (Underwood et al, 1996). Anaphylactic shock is a rapidly, developing severe allergic reaction that may occur in an individual who has been previously sensitized to an antigen is re-exposed to it. The result of this antigen-antibody reaction releases large quantities of histamine, causing widespread capillary permeability (Janeway et al, 2001). Septic shock is due to bacterial toxins that cause vasodilation, thus increasing capillary permeability. Microorganisms including Rickettsia, Filovirus, and Arenavirus also damage endothelial cells, which result in permeability. Rickettsia, a small gram negative bacteria that causes diseases such as Rocky Mountain spotted fever result from the replication of bacteria in endothelial cells with subsequent damage to the cells and blood vessel leakage (Murray et al, 2002). Ebola virus which belongs to the Filoviridae family is an example of an agent that causes severe hemorrhagic fever and causes extensive tissue necrosis in parenchymal cells of the liver, spleen, lung, and lymph nodes, as well as the breakdown of endothelial cells leading to vascular injury (Murray et
For Arenaviruses such as Lassa, which also causes hemorrhagic fever, the pathogenesis involves the release of mediators as well as significant tissue damage.

In summary, vascular permeability can be the result of diverse causes ranging from microorganisms such as bacteria and virus to clinical complications as seen in atherosclerosis. The permeability may be due to soluble mediators (e.g. pre-eclampsia, anaphylactic shock, septic shock) and/or direct damage to the endothelial cells (e.g. Ebola, Lassa, Rickettsia). For those diseases caused mainly by soluble mediators the permeability may be reversed with treatment such as fluid replacement, but in many of the severe cases, the progression and quantity released is so great that the outcome may be quite devastating. Depending upon the severity, disease that directly damage endothelial cells can also lead to fatality as seen in the high mortality rates in Ebola cases (Murray et al, 2002). The unique characteristic of dengue is its lack of endothelial cell damage as observed in DHF/DSS patients, thus postulating that soluble mediators are the more likely cause for pathogenesis rather then direct cytopathologic effects from viral infection.

Since we are living in a time of bioterrorist threats, it is important to be prepared for such an unfortunate event. Viral hemorrhagic fevers are a possible threat in this aspect because no vaccines or therapeutics is available for these potentially fatal diseases. Understanding the mechanism of the pathogenesis as well as the host-virus relationship seen in the “immune enhancement” theory is critical in helping us provide therapeutic solutions. For this project, dengue virus infection will be used as a model for viral hemorrhagic syndromes.
**Specific Aims**

1) Develop an *in vitro* antibody dependent enhancement (ADE) model.

2) Determine whether there is an increase of specific cytokine/chemokine production from infected monocytes.

3) Develop a static *in vitro* vascular endothelial permeability model to evaluate permeabilizing activity of dengue 2-infected cell supernatants.
CHAPTER II
MATERIALS AND METHODS

U937 Monocyte Cell Culture

The human monocytic cell line U937 was grown and maintained in RPMI 1640 medium (Gibco, Carlsbad, CA) containing 1% sodium pyruvate (Sigma, St.Louis, MO), 10% FBS (Hyclone, Logan, UT, Lot Number 19052271A), HEPES (Acros, New Jersey), hypoxanthine (Sigma, St. Louis, MO) and gentamycin (Sigma, St. Louis, MO). Cells were maintained in 24-well, flat bottom plates, as well as 25-cm$^2$ tissue culture flasks (Corning/Costar, Cambridge, MA) and kept at 37°C with 5% CO$_2$. Fresh media was given as needed to maintain viability.

For determining cell counts and viability, an equal volume of cell suspension and trypan blue (0.4% in PBS) was loaded into a hemocytometer and was counted using a light microscope under 25x magnification.

One day prior to running assays, the cells were centrifuged at 3100 rpm for 3 minutes in a 5 ml snap cap after which the old media was decanted and the cells resuspended in fresh media.

C6/36 Cell Culture

C6/36 cell clone of Aedes albopictus was grown and maintained in 25-cm$^2$ tissue culture flasks with Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma, St. Louis MO) supplemented with 1% l-glutamine (Sigma, St. Louis, MO), 1% pyruvate, and 10% FBS in a candle jar kept at 30°C. Fresh media was given as needed to maintain viability.
Adherent cells were detached from the growth surface with 0.25% trypsin-1mM EDTA (Trypsin-EDTA)(Gibco-BRL), resuspended, counted, diluted to the appropriate density in DMEM, and then seeded onto 48- or 6-well plates.

**LLC-MK2 Cell Culture**

Rhesus monkey kidney (LLC-MK2) cells were maintained in RPMI 1640 media supplemented with 5% FBS at 37°C with 5% CO₂. Fresh media was provided every 48 to 96 hours. Cells were grown in 25-cm² tissue culture flasks (Costar, MA). Confluent cells were observed under an inverted light microscope.

To remove the monolayer, 1 ml of trypsin-EDTA (1X) solution (Sigma, St. Louis MO) was added to the flask and shaken every 15 minutes to loosen the cells. Time periods for adequate cell detachment ranged between 1 to 4 hours. To inactivate the trypsin, 2-3 ml of complete media (RPMI with FBS) was added to the suspension, which was then pipetted into a 5 ml snap cap and spun at 3100 rpm for 3 minutes to pellet the cells.

**Dermal Microvascular Endothelial Cell Culture**

Dermal microvascular endothelial cells (DMVEC) were maintained in Human Endothelial SFM Basal Growth media (Gibco) supplemented with 10% human serum, 1% l-glutamine, and 100μg of endothelial cell growth supplement (ECGS) (BD Biosciences). Cells were grown in 25-cm² tissue culture flasks at 37°C with 5% CO₂.

To remove the cells, 1 ml of trypsin-EDTA (1X) solution was added to the flask. Trypsin was inactivated by adding 2 ml of complete Endothelial SFM media with 10%
human serum. This cell suspension was added to a 5 ml snap cap and spun at 3100 rpm for 2 minutes to pellet the cells.

**Virus Propagation**

Banked dengue 2 serotype (strain 16681) was provided by the Department of Tropical Medicine, University of Hawai‘i. It was passaged 8 times in PDK (primary dog kidney) cells and frozen down on October 2, 1980 (Halstead *et al.*, 2003). 24 hours before prior to virus inoculation, a 25-cm² tissue culture flask of confluent C6/36 cells was replenished with fresh media. The media was removed from the C6/36 cells and 0.5mL of the dengue virus was added to the monolayer and manually shaken every 10 minutes during the 30°C 1 hour incubation for maximal viral adsorption. Then 5mL of fresh DMEM media with 10% FBS was added and the infected cells were incubated in a candle jar at 30°C. Culture supernatant was harvested on day 6 and stored at 4°C.

**Plaque Assay**

All plaque assays were performed in 6-well tissue culture plates (Costar, MA) with confluent MK2 cells. Viral dilutions that were plated ranged from $10^{-5}$ to $10^{-8}$ along with a negative control (no virus, media only) and positive control ($10^5$). The media from the plate was removed and the viral dilutions were allowed to adsorb for 90 minutes in a 37°C incubator with 5% CO₂. 2% agarose in TAE (1x) was melted along with an equal volume of complete media RPMI (with 8% FBS with 400 μl of 0.33% neutral red dye in PBS). Both were kept in a hot water bath until the temperature reached 44°C whereupon the agarose and prepared media was mixed and 2 ml of this mixture was aliquoted on top
of each well. The agar was left to solidify then the plate was incubated at 37°C with 5% CO₂. The cells were checked visually the next day to determine that the cells were still viable.

**Cord Blood Serum**

Banked cord blood serum (23785) from a dengue infected mother collected in December of 1979 was provided by the Department of Tropical Medicine, University of Hawai‘i. The antibody titers, hemaglutinin inhibition (HI) and plaque reduction neutralization test (PRNT) assays for this serum against all 4 dengue serotypes were done previously (Halstead *et al*, 1983)(see Table 2 in Results). These studies were reviewed and approved by the University of Hawai‘i Committee on Human Studies.

**Antibody Dependent Enhancement Assay**

The cord blood serum was heat inactivated at 56°C for 30 minutes. Ten-fold dilutions starting from $10^{-1}$ to $10^{-8}$ were initially done to screen for the dilution that would result in the greatest viral enhancement. Two MOI’s (0.1 and 0.05) were initially tested but all assays thereafter were performed at an MOI of 0.05. Experiments were done in triplicate in 48-well tissue culture plates with $5 \times 10^5$ U937 cells per well and incubated at 37°C with 5% CO₂. U937 cells with and without virus were also assayed alongside with the immune serum cultures. Cells were harvested on days 1, 2, 3, 4, and 5 days after virus infection and aliquots of cells was kept for RNA extraction and for flow cytometry assays.
Intracellular Labeling using Flow Cytometry

Flow cytometry was used to detect virus uptake by U937 monocyte cells as well as for cytokine expression by double labeling the cells with fluorescein isothiocyanate (FITC) labeled anti-virus antibodies and phycoerythrin (PE) labeled anti-cytokine antibodies. A monoclonal antibody against dengue virus 2 1B10-3-D was provided by the Department of Tropical Medicine, University of Hawai‘i. This monoclonal antibody was titrated to determine its optimal dilution endpoint using dengue 2 (16681) infected C6/36 cells, which was found to be at 1:500. 2mM monensin was added to U937 cultures 1-2 hours prior to labeling assays were performed. Culture supernatants were kept and stored at -80°C for permeability assays. Permeabilization buffer (0.1% saponin, 1% FBS in PBS) and monoclonal antibody 1B10-3-D diluted in PBS (1:500 dilution) was added to the cells and put on ice for 30 minutes. Then 0.75ml of permeabilization buffer was added and the cells were centrifuged at 3000 rpm for 3 minutes and the supernatant removed. Permeabilization buffer and FITC-labeled rabbit anti-mouse IgG (1:100 dilution, BD Biosciences) was added and incubated in the dark at 4°C for 30 minutes. Permeabilization buffer was added and the cells were centrifuged at 3000 rpm for 3 minutes and the supernatant removed. A final addition of permeabilization buffer with the appropriate PE-conjugated anti-cytokine monoclonal antibody (TNFα, IL-10, IL-8)(Pharmingen) was incubated on ice for 30 minutes. Intracellular staining buffer was added and mixed by inverting the tube several times. The cells were pelleted by centrifugation at 3000 rpm for 3 minutes the supernatant was removed using a Pasteur pipette and the cells were fixed with 1% paraformaldehyde. Cells were brought up in
1ml of DPBS and a minimum of 10,000 cells analyzed on an EPICS-XL-MCL flow cytometer (Coulter, Miami, FL).

**RNA Extraction**

The RNA from uninfected, mitogen-stimulated (LPS), and dengue 2-infected U937 monocytes (with and without immune serum) were extracted using Qiagen’s RNeasy kit (Valencia, CA). The number of U937 cells per test ranged between $3 \times 10^5$ cells/mL to $5 \times 10^5$ cells/mL. The amount of LPS used to stimulate the U937 cells was 1ng/mL and 25ng/mL. RNA was isolated following Qiagen’s RNeasy kit manual.

**cDNA**

Immediately following the extraction of RNA from the U937 cells, it was converted to cDNA by RT-PCR. For each reaction, 15μL of master mix (Promega, Madison, WI) and 5 μL of extracted RNA was added to each PCR tube. The master mix contents includes: MgCl2, 5X M-MLV buffer, 10mM dNTP, RNaisin, MMLV-RT, oligodT primer, and RNase-free water. The program file run on the thermocycler is as follows: 42°C for 15 minutes, 99°C for 5 minutes, 4°C for 5 minutes. The cDNA was then transferred to eppendorf tubes and stored at -80°C.

**Cytokine mRNA Expression**

PCR using cytokine specific primers for IL-10, IL-8, and TNFα was done to detect cytokine mRNA expression of the various U937 cell cultures (Table 1). The PCR reaction per tube contained 2 μL cDNA, 1 μL 5’ primer, 1μL 3’ primer, and 21 μL PCR
Supermix (Invitrogen, Carlsbad, CA). The program file run on the thermocycler is as follows: 94°C for 1 minute, 60°C for 1 minute, 68°C for 1 minute. The PCR reaction was run for 35 cycles.

9μL of PCR product and 1 μL of BPB loading dye were loaded onto a 2% agarose (in 1X TAE) gel and run at 70V for 1 hour. A 50 base pair DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight standard. The gel was photographed and band intensities were semi-quantified using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). Table 1 gives the band size for each cytokine’s PCR product as well as the primer sequences.

Table 1. Band sizes in base pairs of cytokine PCR products and primer sequences.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Band size (base pairs)</th>
<th>Primer sequences (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α</td>
<td>444</td>
<td>F: GAGTGACAAGCCTGTAGCCATGTGTAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCAATGATCCAAAGTAGACCTGCCCAGACT</td>
</tr>
<tr>
<td>IL-10</td>
<td>328</td>
<td>F: AAGCGAGAACCAAGACCCAGACCATCAAGGCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGCTATCCAGACAGCCAGATCCGATTTGG</td>
</tr>
<tr>
<td>IL-8</td>
<td>289</td>
<td>F: ATGACTTCAAAGCTGCGCGTGCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TTCAGCCCTCTTCAAAAAACTTCTC</td>
</tr>
</tbody>
</table>
**Endothelial Cell Monolayer**

Transwell-COL membrane assemblies (Costar, 6.5mm diameter, 0.4mm pore size, collagen coated PTFE membrane) with polystyrene plates were used for the static monolayer model. Dermal microvascular endothelial cells were seeded onto the Transwell membrane by the addition of 100-200μL of cell suspension (1-5 x 10⁵ cells/insert) to the upper chamber. Medium was replaced every 24-72 hours and the monolayer confluency was observed using under an inverted light microscope (Nikon). The integrity of the endothelium barrier was determined by the horseradish peroxidase assay as described below.

**Permeability Assays**

**Albumin flux assay**

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to assess the endothelial cell monolayer integrity by the amount of human albumin crossing the membrane (Jacobs et al, 2002). ELISA plates (NUNC, Denmark) were coated overnight in 4°C with 1:1000 Mouse Anti-Human serum albumin (Zymed, CA) diluted in BBS. Plates were then washed twice with HSBBS. Next for the blocking step, 200μl per well of 1% casein in BBS was added and incubated for 1 hour at 37°C. The plates were then washed 2 times with HSBBS then 1 time with BBS and loaded with 100μl of test sample diluted in RPMI media (Sigma, MO). Samples were plated as duplicates and incubated for 1 hour at 37°C. The plate was washed 3 times with HSBBS/0.05% tween20 then 100μl of 1:1000 peroxidase-conjugated Goat IgG fraction to human albumin antibody (ICN Pharmaceutical, OH) diluted 1:1000 in 0.01% casein in BBS. After incubation for
1 hour at 37°C, the plate was then washed 3 times with HISBBS/0.05% twen20. It was then developed by the addition of 100μL ABTS ([2,2'-AZINO-bis [3-ethylbenziazoline-6-sulfonic acid]]) substrate (Zymed, OH) to each well and left at room temperature for 15 minutes. The optical density at 450nm of each aliquot was read in an automated 75XX series ELISA plate reader (Packard Instrument Company, Meriden, CT).

**Horseradish peroxidase assay**

A horseradish peroxidase (HRP) assay developed by Carr et al was used to assess the endothelial cell monolayer integrity. Once a monolayer was observed to be confluent by microscopy, the HRP assay was used to determine its integrity. 10μg/ml of horseradish peroxidase (HRP) was added to the top chamber containing the monolayer and 20μl aliquots at time points ranging from 2-20 minutes were taken from the bottom chamber. This aliquot was diluted (1:10) with media and developed by the addition of 100μL ABTS ([2,2'-AZINO-bis [3-ethylbenziazoline-6-sulfonic acid]]) substrate (Zymed, OH) and incubated at room temperature for 15 minutes. The optical density at 450nm of each aliquot was read in an automated 75XX series ELISA plate reader (Packard Instrument Company, Meriden, CT).
CHAPTER III
RESULTS

Use of flow cytometry to detect dengue 2 infection of U937 cells

Four previously described monoclonal antibodies against dengue serotype 2 (1B10, 3H1, 3H5, 4G2) were provided by the Department of Tropical Medicine, University of Hawai‘i. These were tested for use in flow cytometry by intracellularly staining dengue 2-infected C6/36 Aedes albopictus cells. This mosquito cell line has been routinely used to propagate dengue viruses (Avirutnan et al., 1998). After 6 days of incubation with dengue 2, the C6/36 cells were analyzed using flow cytometry. Figure 1 shows the percentages of infected cells labeled with the four anti-dengue monoclonal antibodies. 1B10 showed the best staining of dengue 2-infected C6/36 cells. Therefore, 1B10 was used in all subsequent experiments. Figure 2 shows a titration curve for 1B10 to obtain the highest working dilution which identified the maximal percentage of positive cells, which corresponded to a dilution of 1:500. For that reason all future experiments used monoclonal antibody 1B10 at a dilution of 1:500. Figure 3 shows the flow cytometric analysis of uninfected and dengue infected C6/36 cells labeled with 1B10 to obtain gating patterns for further experiments.
Figure 1. Intracellular staining of dengue 2 infected C6/36 A. albopictus cells using four anti-dengue 2 monoclonal antibodies.

Figure 2. Titration of anti-dengue 2 monoclonal antibody 1B10 of dengue 2 infected C6/36 cells for 6 days.
Enhancement assay parameters for U937 monocytic cell line

Antibody dependent enhancement (ADE) of dengue viruses has been described previously by Halstead et al, 1983 and many of the protocols used in those studies were followed as a guide to obtain comparable results in the current project. Banked cord blood serum of an infant born to a dengue-immune mother from the Philippines dengue immune serum 23785 has been previously designated as being “enhancing” in those studies (Halstead et al, 1983). The antibody titers against all 4 dengue serotypes had been determined using HI (hemagglutinin inhibition) and PRNT (plaque reduction neutralizing) tests, shown in Table 2. Flow cytometric determination of viral enhancement of serum 23785 was investigated by diluting the serum ten-fold beginning from $10^{-1}$ to $10^{-8}$ to determine the concentration that would result in peak viral enhancement. Two multiplicities of infection (MOI’s) were tested at $0.1$ and $0.05$. A
representative forward and side scatter analysis of U937 cells is shown in Figure 4 with the population of cells gated on for each experiment (circled as "A") consistent for >80% of the total. Viral enhancement was observed in both MOI experiment and at a number of different serum dilutions (Figure 5) however, the greatest viral enhancement of U937 cells was seen with a serum dilution of $10^{-1}$ and a MOI of 0.05. All subsequent experiments were performed at an MOI of 0.05 and a serum dilution of $10^{-1}$.

Table 2. Characterization of banked cord blood serum 23785 for hemagglutinin inhibition and plaque reduction neutralization of different dengue virus serotypes.

<table>
<thead>
<tr>
<th>Cord #</th>
<th>DV1</th>
<th>DV2</th>
<th>DV3</th>
<th>DV4</th>
<th>DV1</th>
<th>DV2</th>
<th>DV3</th>
<th>DV4</th>
</tr>
</thead>
<tbody>
<tr>
<td>23785</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>10</td>
<td>10</td>
<td>86</td>
<td>&lt;10</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

*Titer measure as reciprocal of end point dilution
Figure 4. Representative forward (FS) and side scatter (SS) analysis of U937 cells. Percentage of cells within gate A is 89.1%. Graph represents an analysis of 10,000 events.

Figure 5. Comparison of MOI and serum dilution for optimal viral enhancement of dengue infection in U937 cells.
Dengue 2 enhanced in U937 cultures with dengue-immune serum 23785

U937 cells infected with dengue 2 at an MOI of 0.05 were incubated with or without serum 23785 (1:10) for 24, 48, 72, 96, and 120 hours to determine titration curves for DV2 infectivity as well as activation of TNFα, IL-8, and IL-10 production. It was found that at each time point, cultures incubated with serum 23785 (1:10) showed an increased number of dengue virus infected U937 cells. This viral enhancement ranged from a 1.79- to 6.6-fold increase in virus-producing cells as compared to cultures incubated without serum with the greatest viral enhancement seen at 24 hours after infection (Figure 6). Figure 7 shows a representative analysis of FITC or dengue-positive cells of dengue 2-infected U937 cultures incubated with and without immune serum for 72 hours. It was found that of the gated cell population infected with virus alone, 3.7% were found positive for dengue 2 and those incubated with serum had 25.4% dengue-positive cells. Therefore, dengue 2 virus incubated with this serum resulted in enhanced dengue infection in U937 cells. Percentages of dengue 2-positive U937 cells for all 5 time points are shown in Table 3. These numbers represent the averages of 3 runs.
Figure 6. Flow cytometric detection of intracellular dengue 2 in infected U937 cells incubated with and without immune serum at 24, 48, 72, 96, and 120 hours.
Figure 7. Comparison of flow cytometric analysis for dengue virus enhancement in U937 cells incubated with and without immune serum 23785 (1:10) at MOI of 0.05 for 72 hours.

- Blue peak: U937 cells incubated with dengue 2 virus only
- Red peak: U937 cells incubated with dengue 2 virus and immune serum 23785 (1:10)
- U937 cells stained with FITC-labeled anti-dengue 2 monoclonal antibodies
Table 3. Percentage of dengue positive U937 cells incubated for 24, 48, 72, 96 and 120 hours with and without serum 23785 detected by flow cytometry.

<table>
<thead>
<tr>
<th>Time:</th>
<th>24HR</th>
<th>48HR</th>
<th>72HR</th>
<th>96HR</th>
<th>120HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937 cells incubated with:</td>
<td>DV2</td>
<td>DV2 + serum</td>
<td>DV2</td>
<td>DV2 + serum</td>
<td>DV2</td>
</tr>
<tr>
<td>% DV2 positive</td>
<td>1.82</td>
<td>12</td>
<td>4.5</td>
<td>17.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Fold increase of DV2 with serum</td>
<td>1</td>
<td>6.6</td>
<td>1</td>
<td>3.9</td>
<td>1</td>
</tr>
</tbody>
</table>

**Cytokine expression of dengue 2-infected cultures with and without dengue-immune serum**

The U937 cultures that were intracellularly labeled for dengue virus (Table 3) were also investigated for levels of IL-8, TNFα, IL-10 cytokine expression. The percentage of IL-8 positive cells was increased in those cultures incubated with immune serum, as shown in Figure 8, which also increased over time up until 96 hours. Among these IL-8 positive cells, majority were dengue-infected, as shown in Figure 9c. For comparison, a scatter graph of cultures infected with virus alone showed 2.59% IL-8 positive cells (Figure 9b). Flow analysis for control cells is shown in Figure 9a. The trend for IL-8 expression extends to all 5 time periods. A slight increase of TNFα was observed in cultures with immune serum incubated for 96 and 120 hours (Figure 10). Majority of the TNFα positive cells were dengue infected. The scatter graph for cultures incubated for 120 hours with serum, double labeled for dengue virus and TNFα, is shown in Figure 11. There was no significant IL-10 expression observed for all 5 time periods.
(Figure 12). The scatter graph for cultures incubated for 120 hours with serum, double labeled for dengue virus and IL-10 is shown in Figure 13. A summary of flow results for all infected cultures with and without serum is shown in Figure 14.

Figure 8. Kinetics of intracellular IL-8 production in dengue-infected U937 cultures incubated with and without immune serum.
Figure 9. Representative flow cytometric analysis of intracellular IL-8 production in U937 cells incubated with dengue 2 alone with serum 23785 (1:10) at MOI of 0.05 for 72 hours.

a) Isotype controls percentage of cells infected with dengue 2 (region 4) 0.66%, percentage of cells producing IL-8 (region 1) 0.82%, percentage of cells infected with dengue 2 and producing IL-8 (region 2) 0.91% b) No serum added, percentage of cells infected with dengue 2 (region 4) 1.4%, percentage of cells producing IL-8 (region 1) 0.68%, percentage of cells infected with dengue 2 and producing IL-8 (region 2) 1.8% c) Serum added, percentage of cells infected with dengue 2 (region 4) 10.1%, percentage of cells producing IL-8 (region 1) 0.39%, percentage of cells infected with dengue 2 and producing IL-8 (region 2) 14.5%. Each graph represents the analysis of at least 10,000 events.
Figure 10. Kinetics of intracellular TNFα production in dengue-infected U937 cultures incubated with and without immune serum.
Figure 11. Representative flow cytometric analysis of intracellular TNFα production in U937 cells incubated with dengue 2 alone with serum 23785 (1:10) at MOI of 0.05 for 120 hours.

a) Isotype controls percentage of cells infected with dengue 2 (region 4) 0.98%, percentage of cells producing TNFα (region 1) 0.65%, percentage of cells infected with dengue 2 and producing TNFα (region 2) 1%
b) No serum added, percentage of cells infected with dengue 2 (region 4) 20.4%, percentage of cells producing TNFα (region 1) 0.06%, percentage of cells infected with dengue 2 and producing TNFα (region 2) 2.07%
c) Serum added, percentage of cells infected with dengue 2 (region 4) 17.9%, percentage of cells producing TNFα (region 1) 0.07%, percentage of cells infected with dengue 2
and producing TNFα (region 2) 4.06%. Each graph represents the analysis of at least 10,000 events.

Figure 12. Kinetics of intracellular IL-10 production in dengue-infected U937 cultures incubated with and without immune serum.
Figure 13. Representative flow cytometric analysis of intracellular IL-10 production in U937 cells incubated with dengue 2 alone with serum 23785 (1:10) at MOI of 0.05 for 72 hours.

a) Isotype controls percentage of cells infected with dengue 2 (region 4) 0.98 %, percentage of cells producing IL-10 (region 1) 0.65%, percentage of cells infected with dengue 2 and producing IL-10 (region 2) 1%
b) No serum added, percentage of cells infected with dengue 2 (region 4) 13.8%, percentage of cells producing IL-10 (region 1) 0.23%, percentage of cells infected with dengue 2 and producing IL-10 (region 2) 1.28%
c) Serum added, percentage of cells infected with dengue 2 (region 4) 29.8%, percentage of cells producing IL-10 (region 1) 0.03%, percentage of cells infected with dengue 2 and producing IL-10 (region 2) 1.75%. Each graph represents the analysis of at least 10,000 events.
Figure 14. Kinetics of intracellular cytokine production in U937 cells positive for dengue virus 2.

**Enhanced dengue uptake not observed in U937 cells when incubated with non-dengue serum**

To ensure the enhancement of dengue virus infection seen in cultures with immune serum 23785 was not due to a non-specific quality of serum, U937 cultures were infected with dengue 2 virus in the presence of normal serum lacking dengue antibodies. U937 cultures were set up exactly the same way as for the enhancement assay, except that normal, non-dengue serum was used instead of immune serum. Enhancement was not observed in those cultures, although the percentage of dengue positive U937 cells in this experiment was significantly lower than previous studies (Figure 15).
Cytokine RNA expression of dengue 2 infected U937 cultures

The RNA of dengue 2-infected U937 cultures incubated for 24, 48, 72, 96 and 120 hours with and without immune serum 23785 as well as uninfected U937 cells were extracted and evaluated for cytokine RNA by RT-PCR. Figure 16 is a photograph of the IL-8 RT-PCR gel scanned for Table 4 showing specific IL-8 PCR products of dengue 2 infected U937 cultures incubated for 24, 96 and 120 hours with and without immune serum. All of the samples exhibited IL-8 RNA expression, though infected cultures were not significantly different than uninfected cultures (Figure 17). The range of RNA expression of uninfected cultures is denoted by the horizontal orange lines. Very little TNFα RNA was observed in infected cultures (Figure 18). There was virtually no production of IL-10 RNA by dengue 2-infected cultures (Figure 19). Table 4 lists the net intensity number of each band for all cultures.
As a positive control, U937 cells were incubated with 1 and 25 ng of LPS for the same period of time and similarly evaluated by RT-PCR. The net band intensity comparison of LPS stimulated cells and dengue infected cells are summarized in Table 5. LPS is known to be a very potent inducer of TNFα RNA, as may be seen in Table 5. It also was effective in stimulating IL-8 RNA production but less effective for induction of IL-10 RNA.
Figure 16. Representative experiment of IL-8 mRNA from dengue 2-infected U937 cells cultured with and without serum 23785.

Lane M: Band size marker  
Lane 1: 24 HR culture with dengue 2  
Lane 2: 24 HR culture with dengue 2 and immune serum  
Lane 3: 96 HR culture with dengue 2  
Lane 4: 96 HR culture with dengue 2 and immune serum  
Lane 5: 120 HR culture with dengue 2  
Lane 6: 120 HR culture with dengue 2 and immune serum
Figure 17. Kinetics of IL-8 RNA expression dengue-infected U937 cells with or without immune serum.

Figure 18. Kinetics of TNFα RNA expression dengue-infected U937 cells with or without immune serum.
Figure 19. Kinetics of IL-10 RNA expression dengue-infected U937 cells with or without immune serum.

Table 4. PCR results using cytokine specific primers. Numbers are the net intensity number generated by Kodak 1D Image Analysis software.

<table>
<thead>
<tr>
<th>Time:</th>
<th>24HR</th>
<th>48HR</th>
<th>72HR</th>
<th>96HR</th>
<th>120HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DV2</td>
<td>DV2 + serum</td>
<td>DV2</td>
<td>DV2</td>
<td>DV2 + serum</td>
</tr>
<tr>
<td>U937 cells incubated with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>0</td>
<td>2412</td>
<td>2626</td>
<td>0</td>
<td>2633</td>
</tr>
<tr>
<td>IL-10</td>
<td>1209</td>
<td>5503</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>9991</td>
<td>7325</td>
<td>15,969</td>
<td>11,347</td>
<td>7069</td>
</tr>
</tbody>
</table>
Table 5. Comparison of cytokine band intensity from dengue 2-infected and LPS-stimulated U937 cells. *Arrows denote band intensity number generated by Kodak ID Image Analysis software

<table>
<thead>
<tr>
<th></th>
<th>IL8</th>
<th>TNFα</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DV2</td>
<td>DV2+serum</td>
<td>LPS</td>
</tr>
<tr>
<td>24HR</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
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<td>48HR</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>72HR</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>96HR</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>120HR</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

*↑: 1-5000 ↑↑: 5-10000 ↑↑↑:10-20000 ↑↑↑↑:>20000

Establishment of in vitro model using HepG2 cells

A monolayer of HepG2 cells grown to confluency in the Transwell system was initially used to test the in vitro model. The integrity of the monolayer was measured by the albumin flux across the monolayer by a capture ELISA developed by Jacobs et al (Figure 20). The increasing divergence between the top and bottom aliquots over time indicates less albumin being able to pass through the monolayer thus becoming more confluent. However, the reproducibility of this assay was poor and the endothelial cell line which would be used for the actual study requires human serum supplement which contains albumin. Therefore, the HRP assay was used in all subsequent integrity experiments.

Endothelial cell permeability induced by dengue culture supernatants

Supernatant from U937 cultures stimulated with LPS for 96 hours was added to the endothelial monolayer because it was found that these cells showed an increase in TNFα RNA expression (Table 5). TNFα is known to increase vascular permeability (Janeway et al, 2001). This resulted in an increase in transfer of HRP across the
monolayer as compared to untreated monolayers, thus indicating increased permeability as shown in Figure 21. To ensure this increase in permeability was not due to LPS itself, LPS with media was added to the Transwell and found not to induce a change in permeability as shown in Figure 22. In the next series of experiments, supernatants of dengue virus-infected U937 cultures were evaluated in this endothelial cell permeability model. Supernatants from dengue infected U937 cultures induced endothelial permeability. The most substantial difference of permeability occurred with supernatants of 48 hour cultures of infected cells. Though supernatants of dengue infected cultures incubated without serum induced permeability, those with dengue virus and immune serum induced a higher level of permeability as shown in Figure 23. Figures 24 thru 26 show the permeability results induced by supernatants from 72, 96 and 120 hour incubated cultures. Since the supernatants of infected cultures may contain virus, virus alone (1x10^5 pfu/ml) was incubated in the transwell for 24 hours and found not to induce significant permeability, as shown in Figure 27. Since the monolayer readings for each transwell differed, it is difficult to compare the data side by side therefore, a permeability coefficient graph was used to make the samples more comparable. The permeability coefficient is the OD reading induced by the supernatant to the OD reading of the monolayer. The compiled ratios of all tested supernatants are shown in Figure 28. The sample that consistently induced the greatest permeability was from the 48 hour incubated cultures with immune serum.
Figure 20. Integrity check of HepG2 monolayer by albumin capture ELISA.
Figure 21. ELISA results of HRP assay for endothelial monolayer integrity and supernatant from 96 hour LPS-stimulated cultures. Supernatant incubated for 24 hours.

Figure 22. ELISA results of HRP assay for endothelial monolayer integrity and LPS with media. LPS with media incubated for 24 hours.
Figure 23. ELISA results of HRP assay a) endothelial monolayer integrity and supernatant from 48 hour dengue 2-infected cultures. b) 48 hour dengue 2-infected cultures with immune serum 23785 (1:10). Supernatants incubated for 24 hours.
Figure 24. ELISA results of HRP assay a) endothelial monolayer integrity and supernatant from 72 hour dengue 2-infected cultures. b) 72 hour dengue 2-infected cultures with immune serum 23785 (1:10). Supernatants incubated for 24 hours.
Figure 25. ELISA results of HRP assay a) endothelial monolayer integrity and supernatant from 96 hour dengue 2-infected cultures. b) 96 hour dengue 2-infected cultures with immune serum 23785 (1:10). Supernatants incubated for 24 hours.
Figure 26. ELISA results of HRP assay a) endothelial monolayer integrity and supernatant from 120 hour dengue 2-infected cultures. b) 120 hour dengue 2-infected cultures with immune serum 23785 (1:10). Supernatants incubated for 24 hours.
Figure 27. ELISA results of HRP assay for endothelial monolayer integrity and dengue 2 alone (1x10^3 pfu/ml). Dengue 2 incubated for 24 hours.

Figure 28. Ratio of supernatant OD/monolayer integrity from all cultures.
CHAPTER IV
DISCUSSION

The pathogenesis of dengue hemorrhagic fever is highly speculative. For over three decades, the major phenomenon believed to cause severe manifestations is known as the antibody-dependent enhancement (ADE) theory. The basis of this hypothesis is that cross-reactive non-neutralizing antibodies elicited from a prior infection bind to but does not neutralize the second dengue virus infection of a different serotype. This antibody-virus complex may be taken up more readily than virus alone via Fc receptors present on monocyte-macrophage cells, increasing viral titer as well as the number of virus-infected cells. ADE has been shown in vitro and in vivo as majority of DHF patients have antibodies to one or more dengue serotypes.

In this study, several methods have been established to study ADE of dengue virus infection in vitro. A flow cytometric assay has been shown successful in measuring the number of virally infected cells and cytokine-producing cells. A microvascular permeability model was developed using dermal microvascular endothelial cells using the Transwell system and HRP to measure monolayer permeability.

First, this study has shown the enhancement of dengue 2 uptake by U937 cells in the presence of a dengue-immune serum using flow cytometry. Enhanced viral uptake was consistent for cultures incubated at each time point examined (24, 48, 72, 96 and 120 hours) in dengue 2 infected U937 cultures incubated with and without immune serum and the percentages of dengue-positive cells increases in time. The fact that this serum was previously tested and shown to enhance dengue uptake in vitro using different methods (Halstead et al, 1983), validates the methodology of this study.
The levels of cytokines and chemokines such as TNFα, IL-8 and IL-10 have been found elevated in DHF patients. Such chemical mediators may induce vascular permeability causing plasma leakage and possibly shock, which is similar to those seen in DHF patients. This study has shown increased numbers of IL-8, less of TNFα and no IL-10 producing cells in cultures infected with dengue 2 and immune serum. There seems to be a correlation between virally infected cells and IL-8 production since the majority of cytokine-producing cells were also dengue virus positive. The percentage of IL-8 positive cells continued to increase for up to 96 hours after virus infection and then decreased by more than 50% at 120 hours, even though the number of infected cells continued to rise over time. This may reflect the kinetics of IL-8 production, since most cytokines increase in response to an appropriate stimulus but eventually decrease as a result of regulatory mechanisms. TNFα, known to induce vascular permeability, did not seem to be the major factor in causing increased permeability in this study. Although some TNFα was produced in dengue-infected cultures incubated with immune serum, there was no direct correlation seen in the permeability assays. Similarly, a previous study by Carr et al showed that TNFα was released by dengue-infected monocyte-derived macrophages but its release did not coincide with peak permeability of endothelial cells.

The number of cytokine-producing cells found by flow cytometry did not correlate with cytokine RNA expression, perhaps cytokine expression in dengue-infected cells may be regulated post-transcriptionally. However, the hierarchy of cytokine RNA was similar to cytokine levels measured by flow cytometry. All infected cultures
exhibited IL-8 expression, both at the RNA and protein level. Very low expression of TNFα and virtually no IL-10 expression were seen in infected cultures.

Culture supernatant of cells infected with dengue 2 and immune serum produced a higher level of monolayer permeability than cells infected with virus alone, in particular the 48-hour incubated cultures. While these cultures contained higher levels of IL-8-producing cells than cultures infected with virus alone, the biologic activity assessed by the in vitro permeability model did not appear to strictly correlate with the proportion of IL-8 producing cells in culture. The culture with the highest percent of IL-8 producing cells (96 hours with immune serum) did not induce much permeability, especially compared to the cultures incubated for 96 hours without serum. Despite a lower level of IL-8 producing cells, both 120-hour incubated cultures, with and without serum produced enhanced permeability, although there was no increase in permeability over time but an immediate and sustained permeability. One possible explanation is that the number of cytokine-producing cells may not directly reflect the amount of cytokine present in culture supernatants. Alternatively, in some cultures permeability may be due to dengue virus alone, which may have an effect on permeability at high titers. However, our studies also showed that dengue virus alone does not appear to directly affect monolayer permeability. While our results indicate that the supernatants of infected cells do have an effect on endothelial monolayer permeability, further studies are needed to enable clear conclusions to be drawn.

Now that methods for studying ADE and permeability in vitro have been developed, additional control and immune sera should be tested to obtain statistically significant data. Additional cytokines and permeability factors produced by infected cells
can be evaluated such as vascular endothelial growth factor (VEGF), which has been shown to regulate IL-8 expression both in vitro and in vivo (Reinders et al, 2003). VEGF is secreted by various cell types, among them is monocytes, and is 50,000 times more potent than histamine in inducing vascular permeability. Upon binding to endothelial cells via its receptors Flt-1 and Flk-1, it stimulates Ras GTPase and eventually MAP kinase, which governs endothelial cells junction proteins such as VE-cadherin and occludin (Kevil et al, 1998). These proteins are essential in cell-cell adhesion. When MAP kinase is activated by VEGF, there seems to be subsequent loss of VE-cadherin and occludin from cell-cell junctions. Thus the reorganization of endothelial cell junctional proteins via VEGF may contribute to the permeability observed in the in vitro monolayer, and possibly in the pathogenesis of DHF. In addition, VEGF is known to induce expression of adhesion molecules in endothelial cells and possibly regulate chemokine production, including IL-8 (Reinders et al, 2003).

Expression of cytokine/chemokine receptors on endothelial cells after incubation with infected cell culture supernatant may affect permeability. In a recent study, IL-8 has been shown to activate endothelial cells by two of its receptors CXCR1 and CXCR2 thru Rho and Rac signaling pathways (Schraufstatter et al, 2001). Initially, IL-8 activates CXCR1, which in turn activates Rho GTPase and induces clustering of E-selectin and adhesion molecules, thus allowing for recruitment of leukocytes. Another IL-8 receptor on endothelial cells CXCR2 upon binding of IL-8 activates Rac GTPase, which leads to cell retraction and gap formation between neighboring cells. This may directly lead to increased permeability of these cell monolayers. Activation of PAK, a downstream target of activated Rac, may also cause endothelial cell contraction. Thus, IL-8 may cause
cytoskeletal rearrangement (F-actin) due to activation of CXCR2 on endothelial cells and change the conformation of cells. In addition, activation of Rac and Rho induce NF-κB transcription factor, which may also activate a variety of gene products that may exert biological effects.

There may be many compounds causing increased vascular permeability and IL-8 may serve only as a surrogate marker for those factors. By repeating the RNA expression experiments, another possible future experimental approach may include the analyses of gene expression using microarrays, which may help determine which sets of genes are induced under different circumstances of dengue infection and identify novel proteins that may have the capability of causing changes in permeability.

In conclusion, this study has shown enhancement of dengue 2 infection of U937 cells in the presence of cord blood serum containing dengue antibodies. With an increase in virally infected cells over time, the supernatants from these cultures did not necessarily induce an increase in permeability. IL-8 production was mainly produced by infected cells and did not have a bystander effect on neighboring cells. The peak of IL-8 production also did not coincide with permeability activity. Therefore, IL-8 may play a role in inducing permeability but the cause of increased vascular permeability in microvascular permeability is most likely caused by multiple factors.
REFERENCES


CDC website: www.cdc.gov/ncidod/dvbid/dengue/


